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Rapid and generic identification of influenza A and other respiratory viruses with mass spectrometry

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The rapid identification of existing and emerging respiratory viruses is crucial in combating outbreaks and epidemics. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a rapid and reliable identification method in bacterial diagnostics, but has not been used in virological diagnostics. Mass spectrometry systems have been investigated for the identification of respiratory viruses. However, sample preparation methods were laborious and time-consuming. In this study, a reliable and rapid sample preparation method was developed allowing identification of cultured respiratory viruses. Tenfold serial dilutions of ten cultures influenza A strains, mixed samples of influenza A virus with human metapneumovirus or respiratory syncytial virus, and reconstituted clinical samples were treated with the developed sample preparation method. Subsequently, peptides were subjected to MALDI-TOF MS and liquid chromatography tandem mass spectrometry (LC–MS/MS). The influenza A strains were identified to the subtype level within 3 h with MALDI-TOF MS and 6 h with LC–MS/MS, excluding the culturing time. The sensitivity of LC–MS/MS was higher compared to MALDI-TOF MS. In addition, LC–MS/MS was able to discriminate between two viruses in mixed samples and was able to identify virus from reconstituted clinical samples. The development of an improved and rapid sample preparation method allowed generic and rapid identification of cultured respiratory viruses by mass spectrometry.

1. Introduction

Q3 Respiratory viruses are a major cause of infections, natural outbreaks, and epidemics. Approximately 200 million cases of viral community-acquired pneumonia occur every year: 100 million in children and 100 million in adults (Ruuskanen et al., 2011). A wide range of viruses, including current circulating subtypes of influenza A virus (influenza A(H1N1)pdm09 and influenza A H3N2), influenza B virus, respiratory syncytial virus (RSV), parainfluenza viruses, adenovirus, and rhinovirus, have been implicated in respiratory tract infections in past decades. In the last ten years, new viruses have emerged as severe acute respiratory syndrome (SARS) virus, Middle East respiratory syndrome (MERS), avian influenza A (H5N1) virus, human metapneumovirus (hMPV), coronaviruses NL63 and HKU1, and human bocavirus. Rapid identification of existing and emerging respiratory viruses is of the utmost importance in combating outbreaks and epidemics, and starting early therapy and prophylaxis. Currently, cell culture, serology, and real-time reverse transcription-polymerase chain reaction (rRT-PCR) are used in virological diagnostics. However, there are a few drawbacks with these techniques: (i) serology and rRT-PCR-based assays are target directed and thus potentially miss non-selected or emerging pathogenic viruses (Binnicker et al., 2013; Yang et al., 2014); (ii) culturing is time consuming; (iii) serology-based assays are not applicable in the acute phase and/or have low sensitivity. Furthermore, multiple tests are needed to detect and subtype mixed infections.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is a generic technique that can rapidly identify cultured microorganisms (Seng et al., 2009; van Veen et al., 2010). The analysis of bacteria by mass spectrometry (MS) has made great progress over the last two decades, and it is

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employed in many hospitals as a rapid and reliable alternative to traditional identification methods. Thus far, mass spectrometry-based methods have not been used for virological diagnostics. However, it has been shown that purified influenza virus particles could be identified with either MALDI-TOF MS or MALDI Fourier Transform Ion Cyclotron Resonance MS (MALDI-FT-ICR MS). Identification was based on the proteolytic digestion of concentrated and purified viral samples and on mass spectrometry analysis of the specific peptide mass profile. The sample preparation methods used in these studies included virus concentration and/or purification with either ultracentrifugation, differential centrifugation, precipitation, filtration, isolation of viral particles or protein(s) with an affinity capture immunoassay or gel electrophoresis (Downard et al., 2009; Schwahn et al., 2009a,b, 2010a,b; Chou et al., 2011; Jang et al., 2011; Nguyen and Downard, 2013; Fernandes and Downard, 2014). These sample preparation procedures were laborious and often time consuming (up to 24 h) and are therefore not applicable for high-throughput virological diagnostics.

The aim of this study was to develop a rapid, generic and robust sample preparation method for MALDI-TOF MS and LC–MS/MS that will enable reliable and fast identification of respiratory viruses. For this purpose, cultured influenza A virus strains and mixed samples of influenza A virus with hMPV or RSV were treated with the developed preparation method. Subsequently, obtained peptides were subjected to MALDI-TOF MS and LC–MS/MS for analyses. The identification of the respiratory viruses was based on peptide sequence differences in abundant viral proteins. To confirm correct identification of peptides by mass spectrometry, the amino acid sequences were compared to the corresponding DNA sequences, obtained by sequencing of the viruses. Finally, to determine the sensitivity of the in-house developed method the titers of cultured viruses were determined with rRT-PCR.

2. Materials and methods

2.1. Ethics statement

All of the clinical influenza strains, anonymized from routine diagnostics, originated from a collection at the University Medical Center Utrecht (UMCU). Collection of the samples and analysis of the isolated virus strains were approved by the local Medical Ethics Committee of the UMCU. The institutional review board (IRB) confirmed (protocol 12/320) that the viral strains were not regarded as patient-owned material; consequently, the use of these strains was not restricted by Dutch law ("Law Medical Scientific Research with People", WMO; art. 1b).

2.2. Viruses

Nasopharyngeal swabs and tracheal aspirates were collected between 2009 and 2011 from patients who were hospitalized with respiratory distress symptoms in the wards of the UMCU (Table S1). The collected samples were cultured immediately in either LLC-MK2 or HEp2 cell lines and were routinely checked for cytopathological effect (CPE) formation. The samples exhibiting specific CPE in 80% of the cells and testing positive for respiratory viruses by real-time PCR were harvested and stored at −80 °C.

Next, eight influenza A H1N1 strains (designated here as BM1456, BM1457, and BM1480 through BM1485) and two influenza A H3N2 strains (designated here as BM1454 and BM1455) were cultured in LLC-MK2 cells in the presence of EMEM (Eagle's minimal essential medium) with trypsin at 37 °C (Table S1). In addition, hMPV (designated here as BM1460) and RSV type A (designated here as BM1450) were cultured in LLC-MK2 cells in the presence of EMEM with trypsin at 37 °C and in HEp2 cells in the presence of DMEM (Dulbecco's modified Eagle medium) with 5% FBS (fetal bovine serum) at 33 °C, respectively (Table S1). Subsequently, the culture supernatants were collected, and the cell debris was removed by centrifugation (239 × *g*, 10 min). Aliquots were frozen at −80 °C and were used without further purification steps.

2.3. Viral RNA extraction, cDNA synthesis, and rRT-PCR

To determine the sensitivity of the in-house developed sample preparation method the titers of cultured viruses were determined with rRT-PCR by using a standard curve. This standard curve was developed by counting virus particles using electron microscopy and by subsequently performing PCR.

Viral RNA extraction and PCR were performed according to previously described protocols (Tan et al., 2012). In short, viral genomic RNA was isolated using a MagnaPure LC total nucleic acid kit, according to the manufacturer's guidelines (Roche Diagnostics, Mannheim, Germany). Murine encephalomyocarditis virus was used as an internal control. Reverse transcription of the isolated viral RNA was performed using a MultiScribe reverse transcriptase kit and random hexamers (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's guidelines. PCR primers and probes were designed on the basis of highly conserved genomic regions of the M1 gene for influenza A and the N gene for both hMPV and RSV A, and these primers and probes were used for the typing of the viral strains. The primers and probes used are listed in Table S2.

cDNA samples were analyzed in a 25 μl reaction mixture, containing 10 μl of cDNA, TaqMan universal PCR master mix (Applied Biosystems, ABI), primers and fluorogenic probes labeled with the 5′ reporter dye 6-carboxy-fluorescein (FAM), and the 3′ quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). Amplification and detection were performed with an ABI 7500 system for 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 sec at 95 °C and 1 min at 60 °C. The samples were assessed for the presence of possible inhibitors of the amplification reaction using the indicated internal control, the signals of which had to range within a clear-cut interval.

2.4. Viral genomic cDNA synthesis and sequencing

To confirm the presence and amino acid sequence accuracy of the identified peptides, six of the clinical isolates were sequenced. The RSV strain (BM1450) with accession number JQ901450 was sequenced previously (Tan et al., 2012). For sequencing purposes, hMPV (BM1460) and influenza (BM1454, BM1456, BM1457, BM1480, and BM1483) PCR fragments were obtained by fractional amplification of MagNAPure LC genomic RNA isolates, using the Superscript III one-step RT-PCR System with Platinum Taq High Fidelity kit (Invitrogen) and a 9800 Fast thermal cycler (ABI), according to the manufacturers' protocols. Unlike hMPV and RSV, influenza virus contains a segmented genome consisting of 8 segments. All of the segments were completely amplified and purified from an agarose gel prior to fractional amplification, as previously described (Zhou et al., 2009). The PCR products were applied to a 1% agarose gel and were purified from the gel with a gene jet gel extraction kit (Thermo Fisher Scientific, Landsmeer, the Netherlands), according to the manufacturer's protocol. The isolated fragments were used for whole-genome sequencing.

The hMPV (BM1460) and influenza strains (BM1454, BM1456, BM1457, BM1480, and BM1483) listed in Table S1 were sequenced according to the whole-genome sequence protocol described recently, using the conventional Sanger technique (Tan et al., 2012). Fragments ranging between 650 and 1400 nucleotides were sequenced with an ABI 3730 48-capillary DNA analyzer, using Big-Dye Terminator 3.1 (ABI). The resulting sequence information was assembled into an hMPV whole-genome sequence through alignment with the corresponding reference sequence (GenBank: FJ168779; http://www.ncbi.nlm.nih.gov/genbank/index.html)

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